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METHODS OF SAMPLING AND TEST FOR INGREDIENTS USED IN MEDIA FOR MICROBIOLOGICAL WORK

UDC 576·8 : 620·113

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Alterations

(*Page 9, clause 7.1, line 1*) - Substitute 'water till free from chloride ignite,' *for* 'water, ignite,'.

(*Page 15, clause 11.1, line 1*) - Substitute 'sample' *for* 'meat or the meat product'.

(AFDC 36)

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Indian Standard

METHODS OF SAMPLING AND TEST FOR INGREDIENTS USED IN MEDIA FOR MICROBIOLOGICAL WORK

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(*Continued on page* 2)

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CONTENTS

PAGE

0. FOREWORD ... 4
1. SCOPE ... 4
2. QUALITY OF REAGENTS ... 4
3. SAMPLING ... 5
4. DETERMINATION OF MOISTURE ... 7
5. DETERMINATION OF TOTAL SOLIDS ... 7
6. DETERMINATION OF TOTAL ASH ... 8
7. DETERMINATION OF ACID-INSOLUBLE ASH ... 8
8. DETERMINATION OF AMINO-ACID NITROGEN ... 9
9. DETERMINATION OF TOTAL NITROGEN ... 9
10. DETERMINATION OF FAT CONTENT ... 13
11. DETERMINATION OF SODIUM CHLORIDE ... 15
12. LIMIT TEST FOR ARSENIC (MODIFIED GUTZEIT METHOD) ... 17
13. LIMIT TEST FOR IRON ... 20
14. LIMIT TEST FOR LEAD ... 21
15. DETERMINATION OF COPPER ... 22
16. DETERMINATION OF CHROMIUM ... 25
17. DETERMINATION OF TIN ... 26
18. DETERMINATION OF ZINC ... 28
19. GENERAL GUIDELINES FOR TESTING THE ABILITY TO SUPPORT THE GROWTH OF MICRO-ORGANISMS ... 32
20. CHECKING OF ABSENCE OF FERMENTABLE CARBOHYDRATES ... 33

Indian Standard

METHODS OF SAMPLING AND TEST FOR INGREDIENTS USED IN MEDIA FOR MICROBIOLOGICAL WORK

0. FOREWORD

0.1 This Indian Standard was adopted by the Indian Standards Institution on 28 February 1973, after the draft finalized by the Food Hygiene, Sampling and Analysis Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 A series of Indian Standard specifications is being evolved on various ingredients used in microbiological work. In order to judge the quality of these ingredients, it is essential that uniform methods of sampling and test are employed for assessing the various characteristics of the ingredients. Therefore, with a view to enabling the assessment of some of the common characteristics, this Indian Standard has been formulated. However, the Indian Standard specifications for individual ingredients shall include methods of test that are specific to these ingredients.

0.3 In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS : 2-1960*.

1. SCOPE

1.1 This standard prescribes the methods of sampling and common tests for ingredients used in media for microbiological work.

2. QUALITY OF REAGENTS

2.1 Unless specified otherwise, pure chemicals and distilled water (*see* IS : 1070-1960†) shall be employed in tests.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the test results.

---

*Rules for rounding off numerical values (*revised*).

†Specification for water, distilled quality (*revised*).

3. SAMPLING

3.1 General Requirements of Sampling

3.1.0 In drawing, preparing, storing and handling test samples, the following precautions and directions shall be observed.

3.1.1 Samples shall be taken in a protected place not exposed to damp air, dust or soot.

3.1.2 Precautions shall be taken to protect the samples, the material being sampled, the sampling instrument and containers for samples from adventitious contamination.

3.1.3 To draw a representative sample, the contents of each containers selected for sampling shall be mixed as thoroughly as possible by suitable means.

3.1.4 The samples shall be placed in clean, dry and air-tight glass containers or other suitable containers on which the material has no action.

3.1.5 Each sample container shall be sealed air-tight with a stopper after filling, and marked with full details of sampling, the date of sampling, and the batch and code number.

3.1.6 Samples shall be stored in a manner that the temperature of the material does not vary unduly from the normal atmospheric temperature.

3.2 Scale of Sampling

3.2.1 *Lot* — All the containers of the same size in a single consignment of the material drawn from a single batch of manufacture shall constitute a lot. If a consignment is declared or known to consist of different batches of manufacture, the containers belonging to the same batch shall be grouped together and each group shall constitute a lot.

3.2.1.1 Samples shall be tested for each lot for ascertaining the conformity of the material to the requirements of the specifications. The total number of containers to be selected from the lot shall depend on the size of the lot and shall be in accordance with col 1 and 2 of Tables 1 and 2.

3.2.2 These containers shall be chosen at random from the lot and for this purpose IS : 4905-1968* shall be used. In case such a table is not available the following procedure may be adopted.

3.2.3 Starting from any container, count in one order as 1, 2,......, etc, up to r and so on where r is the integral part of N/n. Every rth container so counted shall be withdrawn to constitute the sample.

---

*Methods for random sampling.

TABLE 1 SAMPLING OF CONTAINERS OF NET CONTENT LESS THAN 300 g

(*Clause* 3.2.1.1)

Number of Containers in the Lot N (1)	Total Number of Containers to be Selected n (2)
9 to 100	9
101 „ 300	12
301 „ 500	15
501 and above	21

TABLE 2 SAMPLING OF CONTAINERS OF NET CONTENT 300 g AND MORE

(*Clause* 3.2.1.1)

Number of Containers in the Lot N (1)	Total Number of Containers to be Selected n (2)
Up to 50	2
51 „ 100	3
101 „ 300	4
301 „ 500	5
501 and above	7

3.3 Test Samples and Referee Sample

3.3.1 *Preparation of Test Sample when the Containers are Selected According to Table* 1 — The sample containers selected according to **3.2.2** and col 1 and 2 of Table 1 shall be divided at random into three equal sets and labelled with the particulars given in **3.1.5**. One of these sets of sample containers shall be for the purchaser, another for the vendor and the third for the referee. The referee samples shall bear the seals of the purchaser and the vendor and shall be kept at a place agreed to between the two.

3.3.2 *Preparation of Test Sample when the Containers are Selected According to Table* 2 — The contents of each of the selected containers, selected according to **3.2.2** and col 1 and 2 of Table 2, shall be mixed thoroughly. With the help of a suitable sampling instrument about 300 g of the material shall be taken and divided into three equal parts. Each part so obtained, shall be transferred to a sample container which shall be air-tight and labelled with the particulars given in **3.1.5**. The samples so obtained shall be divided into three sets in such a way that each set has a sample representing each selected container. One of these sets shall be marked for the purchaser, another for the vendor and the third for the referee. The referee sample shall bear the seals of the purchaser and the supplier and shall be kept at a place agreed to between the two.

3.4 Number of Tests and Criteria for Conformity

3.4.1 Tests for various characteristics (specified in individual specifications) shall be conducted individually on each of the set of samples as obtained in **3.3.1** or as in **3.3.2**.

3.4.2 The lot shall be considered as conforming to the requirements of a specification if each of the individual sample satisfies all the tests.

4. DETERMINATION OF MOISTURE

4.1 Apparatus

4.1.1 *Drying Oven* — capable of operating at 103 ± 2°C.

4.1.2 *Flat-Bottom Dish* — made of nickel or some other suitable material, 7 to 8 cm in diameter, not more than 2·5 cm deep.

4.2 Procedure — Weigh accurately about 2 g of the material in the tared dish. Place the dish containing the sample in the oven maintained at 103 ± 2°C. Remove the dish from the oven after four hours. Cool in a desiccator and weigh. Dry again for 30 minutes, cool and weigh. Continue until two successive weighings do not differ by more than 1 mg.

4.3 Calculate the loss on drying, percent by mass.

5. DETERMINATION OF TOTAL SOLIDS

5.1 Apparatus

5.1.1 *Flat-Bottom Dish* — made of nickel or some other suitable material not affected by boiling water, 7 to 8 cm in diameter and not more than 2·5 cm deep, provided with a short glass stirring rod having a widening flat end.

5.1.2 *Sand* — which shall pass through 500-micron IS Sieve and shall be retained on 180-micron IS Sieve. It shall be prepared by digestion with concentrated hydrochloric acid, followed by thorough washing with water till free from chlorides. It shall then be dried and ignited to full red heat.

5.1.3 *Vacuum Oven*

5.2 Procedure — Heat the dish containing about 20 g of the prepared sand and a stirring rod in the oven for about one hour. Allow to cool in an efficient desiccator for 30 to 40 minutes. Weigh accurately about 2 g of the material into the tared dish. Add about 5 ml of distilled water in the dish and thoroughly mix the sand with the sample by stirring with a glass rod, smoothing out lumps and spreading the mixture over the bottom of the dish.

5.2.1 Place the dish on a boiling water-bath for 30 minutes, then wipe the bottom of the dish and transfer it, along with the glass rod, to the vacuum oven maintained at a temperature between 60 and 70°C and at a pressure of not more than 50 mm Hg.

5.2.2 After two hours, remove the dish to a desiccator, allow to cool and weigh. Replace the dish in the oven for a further period of one hour, remove to the desiccator, cool and weigh again. Repeat the process of heating, cooling and weighing after every one hour till consecutive weighings do not differ by more than 0·5 mg.

5.3 Calculation — Total solids, percent by mass $= \dfrac{100\,(M - M_2)}{M_1 - M}$

where

M = mass in g of the empty dish with the sand and the glass rod,

M_2 = mass in g of the contents of the dish after drying, and

M_1 = mass in g of the contents of the dish before drying.

6. DETERMINATION OF TOTAL ASH

6.1 Procedure — Weigh accurately about 3 g of the material in a tared crucible, ignite at about 550°C, not to exceed very dull redness, until free from carbon, cool in a desiccator, and weigh. If a carbon-free ash is not obtained, wet the charred mass with hot water, collect the insoluble residue on an ashless filter paper, and ignite the residue and filter paper until the ash is white or nearly so. Finally, add the filtrate, evaporate it to dryness, and heat the whole to a dull redness. If a carbon-free ash is still not obtained, cool the crucible, add 15 ml of alcohol, break up the ash with a glass rod, then burn off the alcohol, again heat the whole to a dull redness, cool and weigh. Calculate the percentage of ash from the mass of sample taken.

7. DETERMINATION OF ACID-INSOLUBLE ASH

7.1 Procedure — Boil the ash obtained in **6.1** with 25 ml of diluted hydrochloric acid (1 : 2·5) for five minutes. Collect the insoluble matter on a tared Gooch crucible or ashless filter, wash with hot water, ignite, and weigh. Calculate the percentage of acid insoluble ash from the mass of the sample taken.

8. DETERMINATION OF AMINO-ACID NITROGEN

8.0 Principle — Protein hydrolysates, yeast extract, etc, contain a large proportion of amino-acid nitrogen, that is, nitrogen, that forms part of the terminal $—NH_2$ groups found in amino-acids. The procedure given is known as Sorenson's method. Formaldehyde is used to remove basicity of the $—NH_2$ groups, and the acidity due to the $—COOH$ groups (usually associated with the $—NH_2$ groups) in amino-acids may thus be titrated.

8.1 Reagents

8.1.1 *Standard Sodium Hydroxide Solution* — 0·714 3 N.

8.1.2 *Phenol Red* — Dissolve 0·2 g phenol red in one litre of water.

8.1.3 *Phosphate Buffer Solution* — *p*H 8·0 or use a comparator tube.

8.1.4 *Formaldehyde Solution* — 40 percent of formaldehyde in water.

8.2 Procedure — Shake a suitable known quantity of sample in a conical flask with 100 ml distilled water. Allow to stand for one hour, shaking occasionally. Centrifuge or filter, add a few drops of phenol red, and titrate 10 ml of the filtrate with sodium hydroxide solution to *p*H 8·0. Use the phosphate buffer solution, together with a few drops of phenol red as a colour standard to indicate the correct end point. Add 8 ml of formaldehyde solution. Allow to stand for 15 minutes and titrate again to *p*H 8·0 (let this reading be A ml). Now take 8 ml of the formaldehyde solution and add to 10 ml of water. Titrate this mixture to *p*H 8·0 in the presence of phenol red as before (let this reading be B ml).

8.3 Calculation — Amino-acid nitrogen, percent by mass $= \dfrac{A - B}{10\,M}$

where

M = mass in g of the material taken for the test.

9. DETERMINATION OF TOTAL NITROGEN

9.1 Principle — The product is digested with concentrated sulphuric acid, using copper (II) sulphate as a catalyst, to convert organic nitrogen into ammonium ions. Alkali is added, liberated ammonia is distilled into an excess of boric acid solution, titration is done with hydrochloric acid to determine the ammonia bound with the boric acid, thus the nitrogen content of the sample is calculated from the amount of ammonia produced.

9.2 Apparatus

9.2.1 *Analytical Balance*

9.2.2 *Effective Suction Device* — for the acid fumes evolved during the digestion.

9.2.3 *Grease-Proof Paper* — pieces, about 90 × 60 mm size.

9.2.4 *Heating Device* — on which the Kjeldahl flask can be heated in an inclined position in such a way that the source of heat only touches that part of the flask wall which is below the liquid level. For gas heating, a suitable device is a plate of asbestos provided with a circular hole, so that only the lower part of the flask is exposed to the free flame.

9.2.5 *Kjeldahl Flask* — of not more than 800 ml capacity and provided, if desired, with a pear-shaped glass bulb loosely fitted on top of the neck.

9.2.6 *Distillation Apparatus* — steam or ordinary.

9.3 Reagents

9.3.1 *Boiling Regulators*

9.3.1.1 *For digestion* — glass beads, silicon carbide or splinters of hard porcelain.

9.3.1.2 *For distillation* — silicon carbide or freshly ignited pieces of pumice stone.

9.3.2 *Boric Acid Solution* — Dissolve 40 g of boric acid (H_3BO_3) in water and dilute to 1 000 ml.

9.3.3 *Copper (II) Sulphate* ($CuSO_4.5H_2O$)

9.3.4 *Hydrochloric Acid* — 0·1 N standard volumetric solution, the normality being recorded to four places of decimal.

9.3.5 *Indicator Solution* — In order to avoid deterioration in storage, the quantity of the indicator solution should be prepared according to requirements. Mixed indicator (methyl red and methylene blue), is prepared by dissolving 2 g of methyl red and 1 g of methylene blue in 1 000 ml of ethanol, 95 percent (*v/v*). The colour change of this indicator solution occurs at *p*H 5·4. Store the indicator solution in a brown bottle in a dark and cool place.

9.3.6 *Potassium Sulphate* (K_2SO_4) — anhydrous.

9.3.7 *Sodium Hydroxide Solution* — nitrate free, containing 330 g of sodium hydroxide per 1 000 g of solution.

9.3.8 *Sulphuric Acid* — sp gr 1·84.

9.4 Procedure

9.4.1 *Preparation of Sample* — Proceed with a representative sample of at least 200 g. Make the sample homogeneous. Keep it in a completely filled air-tight closed container and store it in such a way that deterioration and change in composition are prevented. Analyze the sample as soon as possible, but in any case within 24 hours.

9.4.2 Place a few boiling regulators into the Kjeldahl flask, then add about 15 g of anhydrous potassium sulphate and 0·5 g of copper (II) sulphate. Weigh to the nearest 0·001 g about 2 g of the prepared sample (or 1·5 g in the case of a sample rich in fat) on a piece of grease-proof paper. Transfer the grease-proof paper and the test portion to the Kjeldahl flask. Add to the Kjeldahl flask 25 ml of sulphuric acid. Mix by gently swirling the liquid. If desired, a pear-shaped glass bulb may be inserted into the neck of the flask with its tapering end downwards. Place the flask in an inclined position (at an angle of about 40° from the vertical position) on the heating device. At first, heat the flask gently until the foaming has ceased and the contents have become completely liquefied. Then digest by boiling vigorously occasionally rotating the flask, until the liquid has become completely clear and of a light blue-green colour; keep the liquid boiling for another one-and-a-half hours. The total digestion time should not be less than two hours. Take care that no condensed liquid runs down the exterior of the flask. Prevent the escape of too much sulphuric acid caused by overheating during the digestion, because this results in loss of nitrogen. Cool to about 40°C and add cautiously about 50 ml of water. Mix and allow to cool. Pour into a conical flask, of capacity about 500 ml, 50 ml of the boric acid solution from a measuring cylinder, add four drops of the indicator solution, mix and place the flask under the condenser of the distillation apparatus so that the outlet of the adapter dips into the liquid.

Treat the contents of the Kjeldahl flask in one of the following ways:

a) *In the case of steam distillation* — Transfer the contents of the Kjeldahl flask to the distillation apparatus and rinse the flask with about 50 ml of water. Add 100 ml of the sodium hydroxide solution by means of a measuring cylinder. Heat the alkaline liquid by passing steam through it until boiling and keep it so for 20 minutes. At first heat gently to reduce foaming. The volume of collected distillate should be at least 150 ml.

b) *In the case of ordinary distillation* — Cautiously dilute the contents of the Kjeldahl flask with about 300 ml of water and swirl. If desired, transfer to a 1-litre flask. After about 15 minutes add 100 ml of the sodium hydroxide solution carefully down the neck of the flask by means of a measuring cylinder. Immediately attach the flask to the splash-head of the distillation apparatus. Gently swirl the flask to mix the contents. Distil at least 150 ml of liquid, even if the mixture bumps irregularly. Continue the distillation until the mixture starts bumping or 250 ml of distillate has been collected. Make sure that the distillate is cooled effectively and prevent the boric acid solution from becoming warm.

In either case, lower the conical flask just before terminating the distillation, so that the outlet of the adapter is above the liquid level. Rinse the outlet of the adapter above the liquid (internally and externally) with a little water. Verify the completion of the ammonia distillation with a red litmus paper wetted with water; its colour should not be affected by the liquid dripping from the condenser. Stop heating. If the distillation is found to be incomplete, carry out a new determination, carefully following the instructions. Titrate by means of a 50-ml burette, the contents of the conical flask with hydrochloric acid solution. Record, to the nearest 0·02 ml, the number of millilitres of hydrochloric acid solution required. Carry out two determinations on the same prepared sample.

NOTE — The determinations should be carried out in a room free from ammonia vapours.

9.4.3 *Blank Test* — Always perform a blank test (in duplicate) when fresh batches of reagents or freshly prepared solutions are used. It is advisable to carry out a blank test occasionally for reagents and solutions which have already been in use for some time. Carry out this blank test by the procedure described in **9.4.2** taking one piece of grease-proof paper only.

9.5 Calculation

9.5.1 The nitrogen content of the sample,

$$\text{percent by mass} = \frac{1{\cdot}4\ (V_1 - V_0)\ N}{M}$$

where

V_1 = volume in ml of hydrochloric acid solution required for the test,

V_0 = volume in ml of hydrochloric acid solution required for the blank test,

N = normality of hydrochloric acid solution, and

M = mass in g of the test.

9.5.2 If the difference between the result of two determinations, carried out almost simultaneously or in rapid succession by the same analyst, does not exceed 0·10 g of nitrogen per 100 g of sample, report the mean of two results as nitrogen content, percent by mass, to the nearest 0·01 g.

9.5.3 If, in addition to the result in terms of nitrogen, it is desired to express the result as protein, it is necessary to indicate the factor used.

10. DETERMINATION OF FAT CONTENT

10.1 Principle — Boiling of the test portion with dilute hydrochloric acid to free the occluded and bound lipid fractions, filtration of the resulting mass, drying of the fat retained on the filter and extraction with *n*-hexane or light petroleum.

10.2 Apparatus

10.2.1 *Analytical Balance*

10.2.2 *Continuous or Semi-continuous Extraction Apparatus* — Soxhlet type with an extraction flask of about 150 ml.

10.2.3 *Clock Glass or Petri Dish* — diameter not less than 8 cm.

10.2.4 *Cotton Wool* — defatted.

10.2.5 *Desiccator* — containing an efficient desiccant.

10.2.6 *Electrically Heated Drying Oven* — adjusted to operate at 103 ± 2°C.

10.2.7 *Electrically Heated Sand-Bath or Water-Bath or Similar Suitable Apparatus*

10.2.8 *Extraction Thimble* — made of filter paper and defatted.

10.3 Reagents

10.3.1 *Blue Litmus Paper*

10.3.2 *Hydrochloric Acid* — approximately 4 N solution. Dilute 100 ml of concentrated hydrochloric acid (sp gr 1·19) with 200 ml of water and mix.

10.3.3 *n-Hexane or Light Petroleum* — distilling between 40 and 60°C, and having a bromine value less than one. For either solvent, the residue on complete evaporation should not exceed 0·002 g per 100 ml of the solvent.

10.4 Procedure

10.4.1 *Preparation of Sample* — Proceed from a representative sample of at least 200 g. Render the sample uniform by thorough mixing. Keep it in a completely filled air-tight container and store it in such a way that deterioration and change in composition are prevented. Analyze the sample as soon as possible, but in any case within 24 hours.

10.4.2 Weigh 3 to 5 g of the minced sample to the nearest 0·001 g into a 300-ml conical flask. Dry the flask of the extraction apparatus, provided with some boiling chips, for one hour at 103 ± 2°C in the drying oven, allow the flask to cool to room temperature in the desiccator and weigh to the nearest 0·001 g.

10.4.3 Add to the test portion 50 ml of the hydrochloric acid and cover the conical flask with a clock glass. Heat the conical flask on an asbestos wire gauze by means of a gas burner until the contents begin to boil. Continue boiling with a small flame for one hour and shake occasionally. Add 150 ml of hot water. Moisten a fluted filter paper held in a glass funnel with water and pour the hot contents from the flask on to the filter. Wash the flask and the clock glass thoroughly three times with hot water and dry in the oven. Wash the filter with hot water until the washings do not affect the colour of the blue litmus paper. Put the filter paper on the clock glass or petri dish and dry for one hour in the oven at 103 ± 2°C. Allow to cool. Roll up the filter paper and insert it into the extraction thimble. Remove any traces of fat from the clock glass or petri dish, using cotton wool moistened with the extraction solvent, and also transfer the cotton wool to the thimble. Place the thimble in the extraction apparatus. The paper should be handled either with tongs that can be rinsed or with paper cover slips on the fingers. Pour the extraction solvent into the dried flask of the extraction apparatus. Wash the inside of the conical flask used for the disintegration with hydrochloric acid, and the covering clock glass, with a portion of the extraction solvent and add it to the extraction flask. The total solvent quantity should be one-and-a-half to two times the capacity of the extraction tube of the apparatus. Fit the flask to the extraction apparatus. Heat the extraction flask for four hours on hot sand-bath or water-bath. After extraction, take the flask containing the liquid from the extraction apparatus and distil off the solvent using the heated sand-bath or water-bath. Evaporate the last traces of the solvent on the water-bath, using air blowing, if desired. Dry the extraction flask for one hour in the drying oven at 103 ± 2°C and, after allowing to cool to the room temperature in the desiccator, weigh to the nearest 0·001 g. Repeat this process until the results of two successive weighings do not differ by more than 0·1 percent of the sample weighed. Verify the completion of the extraction by taking a second extraction flask and extracting for a further period of one hour with a fresh portion of the solvent. The increase in mass should not exceed 0·1 percent of the mass of the sample. Carry out two determinations on the same prepared sample.

10.5 Calculation

10.5.1 The total fat content of the sample,

$$\text{percent by mass} = 100 \times \frac{M_2 - M_1}{M_0}$$

where

M_2 = mass in g of the flask with the dried fat,

M_1 = mass in g of the empty extraction flask with boiling chips, and

M_0 = mass in g of the test portion.

10.5.2 If the difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst does not exceed 0·5 g of total fat per 100 g of sample, report the mean of two results as total of fat content, percent by mass.

11. DETERMINATION OF SODIUM CHLORIDE

11.1 Principle — Extraction of the meat or the meat product with hot water and precipitation of the proteins. After filtration and acidification, addition of an excess of silver nitrate solution to the extract, and titration with potassium thiocyanate solution.

11.2 Apparatus

11.2.1 *Volumetric Flask* — 200 ml, one-mark.

11.2.2 *Conical Flask* — about 250 ml.

11.2.3 *Burette* — 25 or 50 ml.

11.2.4 *Pipettes* — 20 ml, one-mark.

11.2.5 *pH Meter*

11.3 Reagents

11.3.1 *Nitrobenzene*

11.3.2 *Nitric Acid Solution* — 4 N; mix 1 volume of concentrated nitric acid (sp gr 1·39 to 1·42) with 3 volumes of water.

11.3.3 *Solution Used for Precipitation of Proteins*

11.3.3.1 *Reagent I* — Dissolve 106 g of potassium ferrocyanide [$K_4Fe(CN)_6.3H_2O$] in water and dilute to 1 000 ml.

11.3.3.2 *Reagent II* — Dissolve 220 g of zinc acetate [$Zn(CH_3COO)_2.2H_2O$] and 30 ml of glacial acetic acid in water and dilute to 1 000 ml.

11.3.4 *Standard Silver Nitrate Solution* — 0·1 N; dry silver nitrate ($AgNO_3$) for two hours at 150°C and allow to cool in a desiccator. Dissolve 16·989 g of the dried salt in water and dilute to 1 000 ml.

11.3.5 *Standard Potassium Thiocyanate Solution* — 0·1 N; dissolve about 9·7 g of potassium thiocyanate (KCNS) in water and dilute to 1 000 ml, Standardize the solution to the nearest 0·000 1 N against silver nitrate, using the solution specified in **11.3.4** and the indicator solution specified in **11.3.7.**

11.3.6 *Sodium Hydroxide* — 1 N solution.

11.3.7 *Ammonium Iron (III) Sulphate* [$NH_4Fe(SO_4)_2 \cdot 12H_2O$] *Saturated Solution in Water*

11.3.8 *Activated Charcoal*

11.4 Procedure

11.4.1 *Preparation of Sample* — Proceed from a representative sample of at least 200 g. Render the sample uniform by thorough mixing. Keep it in a completely filled, air-tight container in such a way that deterioration and change in composition are prevented. Analyze the sample as soon as possible, but always within 24 hours.

11.4.2 *Test Portion* — Weigh, to the nearest 0·001 g, about 10 g of the prepared sample and transfer it quantitatively to a conical flask (**11.2.2**).

11.4.3 *Deproteination* — Add successively 0·5 g of activated charcoal (**11.3.8**) and 100 ml of hot water to the test portion in the flask. Heat the contents of the flask for 15 minutes in a boiling water-bath. Shake the contents of the flask repeatedly. Allow the flask with contents to cool to room temperature, then add successively 2 ml of Reagent I (**11.3.3.1**) and 2 ml of Reagent II (**11.3.3.2**). Mix thoroughly after each addition. By means of the sodium hydroxide solution (**11.3.6**) adjust the *p*H to between 7·5 and 8·3, with the help of the *p*H meter (**11.2.5**). Allow the flask to stand for 30 minutes at room temperature, transfer the contents quantitatively to a 200-ml volumetric flask (**11.2.2**) and dilute to the mark with water. Mix the contents thoroughly and filter through a fluted filter paper.

NOTE — The filtrate may also be used for the determination of the nitrate and nitrite content. If ascorbic acid is present in quantities below 0·1 percent in the sample, or if the extract is to be used only for the chloride determination, the activated charcoal can be omitted in preparing the extract. Furthermore, the *p*H adjustment is not necessary if only a chloride determination is to be performed.

11.4.4 *Determination* — Transfer 20 ml of the filtrate to a conical flask (**11.2.2**) by means of a pipette (**11.2.4**) and add 5 ml of the nitric acid solution (**11.3.2**) and 1 ml of the indicator solution (**11.3.7**) with the help of a measuring cylinder. Transfer 20 ml of the silver nitrate solution (**11.3.4**) to the conical flask by means of the pipette. Add 3 ml of nitrobenzene (**11.3.1**) by means of a measuring cylinder and mix thoroughly. Shake vigorously to coagulate the precipitate. Titrate the content of the conical flask with the potassium thiocyanate solution (**11.3.5**). Record to the nearest 0·02 ml, the volume of the potassium thiocyanate solution required. Carry out two determinations on the same prepared sample.

11.5 Calculation

11.5.1 Chloride content of the sample, expressed as a percentage of sodium chloride by mass, is equal to:

$$0{\cdot}005\,844\,(20 - V) \times \frac{200}{20} \times \frac{100}{M} = 58{\cdot}44\,\frac{2 - 0{\cdot}1\,V}{M}$$

where

V = volume in ml of 0·1 N potassium thiocyanate solution required for the titration, and

M = mass in g of the test portion.

NOTE — If the standard volumetric solution of potassium thiocyanate solution is not exactly 0·100 0 N, a suitable correction factor should be used in calculating the result.

11.5.2 If the difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst does not exceed 0·2 g of sodium chloride per 100 g of sample, report the mean of two results as chloride content, to the nearest 0·05 g, percent by mass of sodium chloride.

12. LIMIT TEST FOR ARSENIC (MODIFIED GUTZEIT METHOD)

12.1 Apparatus — As given in Fig. 1.

12.2 Reagents

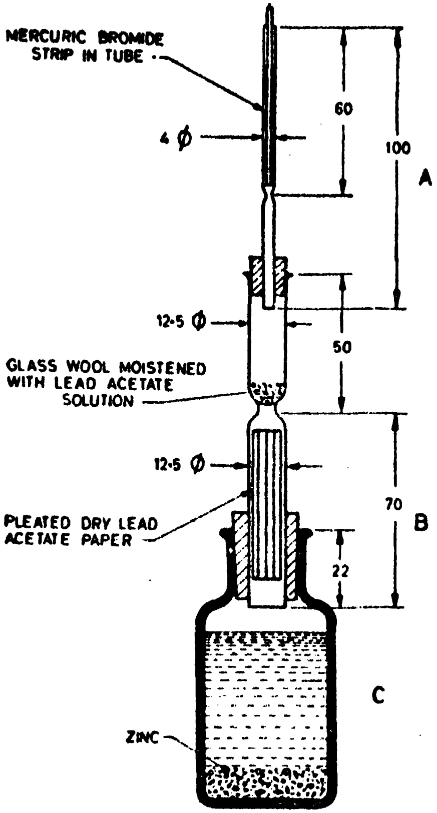
12.2.1 *Lead Acetate Solution* — 10 percent (*m/v*). Add sufficient acetic acid to obtain a clear solution.

12.2.2 *Lead Acetate Paper Strips (Dry)* — Soak filter paper strips cut to a size of 70 × 50 mm in lead acetate solution contained in a glass bottle. Before use, take out the strips and dry in an atmosphere free from hydrogen sulphide.

12.2.2.1 *Absorbent cotton wool impregnated with lead acetate* — Saturate cotton wool with lead acetate solution, drain, press and dry under vacuum at room temperature.

12.2.3 *Mercuric Bromide Solution* — Dissolve 5 g of mercuric bromide in 100 ml of rectified spirit.

12.2.4 *Sensitized Mercuric Bromide Paper Strips* — Soak filter paper strips cut to a size of 70 × 50 mm in mercuric bromide solution contained in an amber coloured glass bottle in dark. Before use, remove the strips, press them between sheets of filter paper, and dry in an atmosphere free from hydrogen sulphide.


MERCURIC BROMIDE STRIP IN TUBE
4 ϕ
60
100
A
12·5 ϕ
50
GLASS WOOL MOISTENED WITH LEAD ACETATE SOLUTION
12·5 ϕ
70
B
PLEATED DRY LEAD ACETATE PAPER
22
C
ZINC

All dimensions in millimetres.

FIG. 1 APPARATUS FOR MODIFIED GUTZEIT TEST OF ARSENIC

12.2.5 *Concentrated Nitric Acid* — sp gr 1·42.

12.2.6 *Sulphuric Acid*—approximately 5 N, and concentrated (sp gr 1·84).

12.2.7 *Hydrochloric Acid* — *see* IS : 265-1962*.

12.2.8 *Potassium Iodide Solution* — 15 percent (*m/v*).

12.2.9 *Stannous Chloride Solution* — Dissolve 40 g of stannous chloride in 100 ml of hydrochloric acid (**12.2.7**). Discard this solution if an appreciable deposit forms.

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*Specification for hydrochloric acid (*revised*).

12.2.10 *Zinc* — granulates of size 0·5 to 1·0 mm; arsenic-free.

12.2.11 *Standard Arsenic Stock Solution* — Weigh, to the nearest 0·1 mg, 0·132 0 g of arsenic trioxide and transfer to a beaker of suitable capacity (100 ml). Dissolve the arsenic trioxide in 2 ml of sodium hydroxide solution prepared by dissolving 50 g of sodium hydroxide in one litre of water. Transfer the solution to a 1 000 ml of volumetric flask. Wash the beaker several times, collect the washings in the volumetric flask and make up the volume. One millilitre of this standard solution contains 100 µg of As.

12.2.11.1 *Standard arsenic solution containing* 2·5 *mg of As per litre* — Take 25 ml of the standard arsenic solution (**12.2.11**), transfer to a 1 000-ml volumetric flask and make up the volume. Prepare this solution at the time of use. One millilitre of this standard solution contains 2·5 µg of As.

12.3 Procedure

12.3.1 *Preparation of the Solution* — Weigh accurately about 50 g of the well mixed sample, transfer to a 500-ml Kjeldahl digestion flask and wet with 25 to 50 ml of concentrated nitric acid. Add 10 to 20 ml of concentrated sulphuric acid and heat cautiously. Add concentrated nitric acid dropwise from time to time from a pipette to speed up the oxidation of the material. Note the total amount of concentrated sulphuric acid and nitric acid added. When the oxidation is complete and the solution is colourless, add 20 ml of water and again boil to fuming. Cool and dilute with water to 50 ml in a graduated flask. Use a suitable aliquot for the estimation of arsenic and reserve the rest for the determination of other heavy metals.

12.3.2 Prepare the solution of the material as specified in the individual material specification. Place the dry lead acetate paper in the lower portion of the tube B and cotton wool moistened with lead acetate in its upper portion. Place the sensitized strip of mercuric bromide paper into tube A and connect the tubes together with a rubber stopper. Introduce a suitable aliquot of the solution of the material into the bottle C and add 10 ml of dilute sulphuric acid. Add 0·5 ml of stannous chloride solution, 5 ml of potassium iodide solution and make up the volume with water to about 50 ml. Mix the contents and drop about 10 g of zinc. Fit in position, the rubber stopper carrying the tube B immediately. Place the bottle in a warm place at about 40°C. Remove the test strips after 40 minutes with the help of tweezers. Conduct the test in exactly similar manner using a volume of standard arsenic solution as specified in the material specification in place of the solution of the material and compare the stain produced with the material with that produced with the arsenic solution.

12.3.2.1 The limit prescribed in the material specification shall be taken as not having been exceeded if the length of the stain as well as the intensity of its colour produced in the test with the material is not greater than those produced with the corresponding standard arsenic solution.

13. LIMIT TEST FOR IRON

13.1 Apparatus

13.1.1 *Nessler Cylinders* — 50 ml capacity, two-matched.

13.1.2 *Platinum Crucible*

13.2 Reagents

13.2.1 *Concentrated Nitric Acid* — sp gr 1·42, free from iron.

13.2.2 *Hydrochloric Acid* — approximately 1 N.

13.2.3 *Hydroxylammonium Chloride Solution* — one percent (*m/v*).

13.2.4 2,2′-*Bipyridyl Solution* — 0·1 percent (*m/v*). Dissolve 0·1 g of the reagent in 50 ml of water containing 2 ml of 1 N hydrochloric acid and dilute to 100 ml with water.

13.2.5 *Ammonium Acetate Solution* — 20 percent (*m/v*).

13.2.6 *Standard Iron Solution* — Dissolve 7·022 g of ammonium ferrous sulphate in a mixture of 600 ml of water and 350 ml of concentrated sulphuric acid (sp gr 1·84). Dilute to 1 000 ml with water and further dilute 10 ml of the solution so obtained to 1 000 ml with water. One millilitre of the diluted solution contains 10 µg of iron. This final solution should be freshly prepared.

13.3 Procedure

13.3.1 Weigh, to the nearest 0·01 g, 2 g of the sample, transfer to the platinum crucible and carefully ignite. Moisten the residue with a few drops of the nitric acid and carefully heat to dryness. Dissolve the residue in about 10 ml of water containing 2 ml of the hydrochloric acid solution and transfer the solution to one of the Nessler cylinders, using about 15 ml of wash water.

13.3.1.1 Add 5 ml of the hydroxylammonium chloride solution and 10 ml of the ammonium acetate solution, mix, add 3 ml of the 2, 2′-bipyridyl solution and dilute to the mark with water. Mix thoroughly and allow to stand for ten minutes.

13.3.1.2 At the same time carry out a comparison test in exactly similar manner using a volume of standard iron solution as specified in the material specification, in place of solution of the material. Compare the colours of the two solutions and note whether the colour of the sample solution is greater than that of the comparison solution.

13.3.2 The limit prescribed in the material specification shall be taken as not having been exceeded if the intensity of its colour produced in the test with the material is not greater than the one produced with the corresponding standard iron solution.

14. LIMIT TEST FOR LEAD

14.1 Reagents

14.1.1 *Citric Acid* — solid.

14.1.2 *Ammonium Hydroxide Solution* — sp gr 0·88 or diluted as required.

14.1.3 *Potassium Cyanide Solution* — 10 percent (*m/v*).

14.1.4 *Dithizone* (*Diphenyl Thiocarbazone*) *Solution* — 0·1 percent (*m/v*) in chloroform, freshly prepared.

14.1.5 *Dilute Hydrochloric Acid* — approximately 0·1 N.

14.1.6 *Standard Lead Solution* — two reference solutions of lead nitrate are required in this test as given under **14.1.6.1** and **14.1.6.2**.

14.1.6.1 *Standard strong lead solution* — obtained by dissolving 0·160 0 g of lead nitrate [$Pb(NO_3)_2$] in 50 ml of dilute nitric acid and making up the volume in a 100-ml graduated flask.

14.1.6.2 *Standard dilute lead solution* — freshly prepared before the test by diluting 1 ml of the standard strong lead solution to 100 ml with water in a graduated flask. One millilitre of this solution contains 0·000 01 g of lead.

14.1.7 *Ammonium Acetate* — solid.

14.1.8 *Sodium Sulphide Solution* — 10 percent (*m/v*).

14.2 Procedure

14.2.1 Take a suitable aliquot of the solution (**12.3.1**), add 2 g of citric acid and just neutralize with ammonia. Add 1 ml of the potassium cyanide solution and transfer the whole to a separating funnel. Extract the liquid with the dithizone solution. Carry out 3 extractions, using 10 ml for the first extraction and 5 ml each for the subsequent extractions. If the last extraction gives any indication of a reddish tinge, extract again to ensure complete removal of lead.

14.2.2 Take 10 ml of water in another separating funnel and wash each extract with this water. If suspended matter is present in the chloroform extract, this shall be filtered before passing to the separating funnel containing the 10 ml of wash water. Transfer the combined chloroform extract to a separating funnel and extract lead by shaking successively with 50 ml, 20 ml and 10 ml of dilute hydrochloric acid. Combine the acid extracts in a separating funnel, wash once or twice with 10 ml of chloroform and filter through a previously wetted filter paper into a 100-ml graduated flask. Make up the volume of the filtrate to 100 ml with dilute hydrochloric acid and use this as the test solution.

14.2.3 Transfer a suitable volume of the test solution to a Nessler cylinder. Add 2 g of ammonium acetate, followed by ammonia until just alkaline and then 1 ml of potassium cyanide solution. Dilute to 50 ml, add 2 drops of sodium sulphide solution and compare the colour produced with the standard dilute lead solution (as specified in the material specification) in the same way.

14.2.4 The limit prescribed in the material specification shall be taken as not having been exceeded if the intensity of its colour produced in the test with the material is not more than the one produced with the corresponding standard lead solution.

15. DETERMINATION OF COPPER

15.1 Methods — Two methods, namely the spectrophotometric (*see* **15.2**) and the gravimetric (*see* **15.3**), are prescribed for the determination of copper in the material. Whereas the spectrophotometric method should be used for reference purposes, for routine analysis the gravimetric method may be used wherever facilities for spectrophotometric analysis are not available.

15.2 Spectrophotometric Method

15.2.1 *Apparatus*

15.2.1.1 *Spectrophotometer* — a suitable type.

15.2.2 *Reagents*

15.2.2.1 *Concentrated sulphuric acid* — sp gr 1·84.

15.2.2.2 *Sodium carbonate* — solid.

15.2.2.3 *Concentrated hydrochloric acid* — sp gr 1·16, diluted with an equal volume of water.

15.2.2.4 *Citric acid* — solid.

15.2.2.5 *Ammonium hydroxide solution* — sp gr 0·90.

15.2.2.6 *Sodium diethyldithiocarbamate solution* — 0·1 percent (*m/v*), aqueous.

15.2.2.7 *Carbon tetrachloride* — redistilled.

15.2.2.8 *Sodium sulphate* — anhydrous.

15.2.2.9 *Concentrated nitric acid* — sp gr 1·42 diluted with an equal volume of water.

15.2.2.10 *Standard copper solution* — Weigh accurately 0·100 0 g of pure copper turning, carefully dissolve in minimum amount of nitric acid, cool and dilute to one litre. Pipette 10 ml of this solution into a 130-ml volumetric flask and dilute to the mark. One millilitre of this solution contains 10 µg of copper.

15.2.3 *Procedure*

15.2.3.1 *Preparation of test solution* — Weigh accurately about 20·0 g of the well mixed sample in a platinum dish and add to it 2 ml of concentrated sulphuric acid. Heat the dish gently over a Bunsen burner until charring is complete and ash the residue in a muffle furnace at 550 to 600°C. Cool the dish, add to the ash about 2 g of sodium carbonate and fuse the contents of the dish ash for 10 minutes at 900°C. Cool the dish and dissolve the fused matter in the minimum amount of hydrochloric acid, covering the dish with a watch-glass to avoid loss by spattering. Heat the dish until solution is complete. Cool the dish and make up the solution to 100 ml in a volumetric flask with water.

15.2.3.2 Transfer 10 ml of the test solution to a separating funnel by means of a pipette. Add one gram of citric acid to the test solution and dissolve it by shaking. Make the resulting solution alkaline to litmus paper by adding ammonium hydroxide solution in small quantities. Add to this alkaline solution, 5 ml of sodium diethyldithiocarbamate solution, shake thoroughly and extract with 5 ml portions of carbon tetrachloride until the final extract is colourless (about four extractions are usually adequate). Dry the combined extracts by shaking thoroughly with anhydrous sodium sulphate. Filter the dry extract and wash the filter paper with carbon tetrachloride. Make up the volume of the filtrate to 25 ml with carbon tetrachloride and measure the absorption at 437 nm by means of the spectrophotometer.

15.2.3.3 Prepare a series of standard colour solutions, using different volumes of standard copper solution instead of 10 ml of the test solution and proceeding as described under **15.2.3.2**. This series should cover the concentration of the colour solutions prepared from the test solution. Measure the absorption of each of the colour solutions in the series.

15.2.3.4 Carry out the blank determinations on water and the reagents used in preparation of the standard colour solutions (*see* **15.2.3.3**) and colour solution from the material (*see* **15.2.3.1** and **15.2.3.2**). If the values so obtained are of any significance, correct the respective values observed for the series of standard colour solution (*see* **15.2.3.3**) and the test solution (*see* **15.2.3.2**) accordingly.

15.2.3.5 Plot a curve using the corrected values for absorption and the corresponding concentration of copper in micrograms present in each standard colour solution in the series. From this curve obtain the mass in micrograms of copper present in 10 ml of the test solution (*see* **15.2.3.1**).

15.2.4 *Calculation*

$$\text{Copper (as Cu) content of the material, } \mu g/kg = \frac{10\,m}{M}$$

where

m = mass in µg of copper present in 10 ml of test solution (*see* **15.2.3.5**), and

M = mass in g of the material taken for the test.

15.3 Gravimetric Method

15.3.1 *Reagents* — The following reagents are required. The reagents shall be free from traces of copper.

15.3.1.1 *Concentrated sulphuric acid* — sp gr 1·84.

15.3.1.2 *Sodium carbonate* — solid.

15.3.1.3 *Concentrated hydrochloric acid* — sp gr 1·16 diluted with an equal volume of water.

15.3.1.4 *Sodium hydroxide solution* — approximately 2 N.

15.3.1.5 *Sodium acetate* — crystalline.

15.3.1.6 *Glacial acetic acid*

15.3.1.7 *Salicylaldoxime solution* — Dissolve 1 g of salicylaldoxime in 5 ml of rectified spirit without the aid of heat. Gently, pour this solution into 95 ml of water at 80°C. The oxime partially separates out in the form of a fine oil suspension, but quickly redissolves. Avoid shaking at this stage as shaking helps the small droplets of the oxime grow. When the solution becomes clear, shake it for some time and filter. Use the filtrate.

15.3.1.8 *Ferrous chloride solution* — about 5 percent (*m/v*).

15.3.2 *Procedure*

15.3.2.1 Proceed as described under **15.2.3.1**, but do not make up the volume to 100 ml.

15.3.2.2 To the whole of the solution so obtained, add sodium hydroxide solution until a lasting precipitate is formed. Add 1 g of sodium acetate and 10 ml of glacial acetic acid, and stir until the precipitate redissolves. Dilute the resulting solution to about 100 ml with water. Add to this dilute solution a bare excess of salicylaldoxime solution to precipitate all the copper present in solution. Coagulate the precipitate by stirring with a glass rod and allow to settle. Test the supernatant liquid for completeness of precipitation by adding several drops of salicylaldoxime solution. (A considerable excess of salicylaldoxime should be avoided, as the precipitate has to be washed free from it before drying. Otherwise the precipitate would be visibly decomposed during drying.) Filter the precipitate through a tared No. 3 Gooch crucible and wash it with cold water until the filtrate ceases to give any colour with ferrous chloride solution. During washing, take care that the precipitate always remains moist. Finally, wash the precipitate twice again with water, and then dry it as far as possible by suction. Dry the crucible with contents to constant mass at 105 to 110°C, cool in a desiccator and weigh. Find the mass of the dried precipitate.

15.3.3 *Calculation*

$$\text{Copper (as Cu), } \mu\text{g/kg} = \frac{189\,200\ m}{M}$$

where

m = mass in g of the dried precipitate as determined under **15.3.2.2**, and

M = mass in g of the material taken for the test.

16. DETERMINATION OF CHROMIUM

16.1 Reagents

16.1.1 *Magnesium Nitrate Solution* — 25 percent (*m/v*).

16.1.2 *Strong Sulphuric Acid Solution* — 4 N.

16.1.3 *Potassium Permanganate Solution* — 0·1 N.

16.1.4 *Sodium Azide Solution* — 5 percent (*m/v*).

16.1.5 *Sodium Dihydrophosphate* — 4 M.

16.1.6 *Diphenyl Carbazide Solution* — Dissolve 125 mg of diphenyl carbazide [(C_6H_5.NH.NH)$_2$CO] in a mixture of 25 ml of acetone and 25 ml of water. This should be prepared immediately before use.

16.1.7 *Standard Chromium Solution* — Dissolve 0·056 g of potassium dichromate ($K_2Cr_2O_7$) in one litre of water. One millilitre of this solution contains 20 µg of chromium.

16.1.8 *Sucrose*

16.2 Procedure — Weigh accurately about 5 g of the material into a quartz or equivalent dish. Char the material, raising the temperature slowly. Allow to cool, add 10 ml of the magnesium nitrate solution and evaporate, heating slowly until no more nitrous vapours evolve. Heat the material in an oven at 600 ± 20°C until all black particles have disappeared (30 to 60 min). Dissolve the residue by adding 10 ml of the strong sulphuric acid solution and 20 ml of water. Heat on a water-bath for about five minutes. Add 0·5 ml of the potassium permanganate solution, cover with a watch glass and heat on a water-bath for about 20 minutes. Add more permanganate, if the solution decolourizes. Add sodium azide solution, one drop after every ten seconds, until the excess potassium permanganate has been removed. (Avoid excess of sodium azide, 2 drops are usually sufficient.) Cool the solution in running water and filter, if manganese dioxide is evident. Transfer the solution to a 50-ml graduated flask. Add 2·5 ml of sodium dihydrophosphate and 2 ml of diphenyl carbazide solution, and fill to the mark with water. Measure the extinction at 540 nm 30 minutes after adding the diphenyl carbazide solution. A blank with the last two reagents (sodium dihydrophosphate and diphenyl carbazide solutions) should show no colour or only a slight purple colour. At the same time, run a parallel test with 1·00 ml of the standard chromium solution and a few milligrams of sucrose placed in a second quartz dish. Treat the mixture exactly as the dye sample and measure the extinction at the same wavelength.

Calculate the chromium content of the dye from the two extinction values observed.

17. DETERMINATION OF TIN

17.1 Reagents

17.1.1 *Concentrated Nitric Acid* — sp gr 1·42.

17.1.2 *Concentrated Sulphuric Acid* — sp gr 1·84.

17.1.3 *Hydrogen Peroxide Solution* — 30 percent by mass.

17.1.4 *Ammonium Oxalate Solution* — saturated, aqueous.

17.1.5 *Ammonium Hydroxide Solution* — sp gr 0·90.

17.1.6 *Concentrated Hydrochloric Acid* — sp gr 1·16.

17.1.7 *Dilute Sulphuric Acid* — 1·3 (*v/v*).

17.1.8 *Hydrogen Sulphide Gas* — from generator, passed through a wash bottle containing water.

17.1.9 *Wash Solution* — Mix 100 ml of saturated ammonium acetate solution with 50 ml of glacial acetic acid and 850 ml of water.

17.1.10 *Ammonium Polysulphide Solution* — Pass hydrogen sulphide gas through 200 ml of ammonium hydroxide solution, contained in a bottle immersed in ice-cold water, until no more gas is absorbed. Add to it 200 ml of ammonium hydroxide solution, and dilute with water to make up to 1 000 ml. Then add 25 g of flowers of sulphur to this solution and keep for several hours to digest the sulphur and then filter.

17.1.11 *Dilute Acetic Acid* — Dilute one volume of glacial acetic acid with nine volumes of water.

17.2 Procedure

17.2.1 *Wet Oxidation* — Weigh accurately 50 to 100 mg of the well mixed sample and place it in a 800-ml Kjeldahl flask. Add to it 25 to 50 ml of concentrated nitric acid and then cautiously add 20 ml of concentrated sulphuric acid. Place the flask on an asbestos mat having a 5 cm diameter hole in the centre. Warm the flask slightly and discontinue heating if foaming becomes excessive. When the reaction subsides, heat the flask cautiously and rotate it from time to time to prevent caking of the material at the bottom of the flask which is exposed to the flame. Maintain the oxidizing conditions in the flask by cautiously adding small quantities of concentrated nitric acid whenever the mixture turns brown or darkens. Continue digestion until all the organic matter is destroyed and sulphur trioxide fumes are copiously evolved. (The final solution should be colourless, or at the most light straw coloured. A persistent yellow colour in the liquid may be discharged by the addition of hydrogen peroxide solution and heating again.) Slightly cool the flask and add 75 ml of water and 25 ml of ammonium oxalate solution to assist in expelling oxides of nitrogen from the solution. Evaporate the solution again to a point where fumes of sulphur trioxide appear in the neck of the flask and then cool it.

17.2.2 *Determination* — Add 200 ml of water to the contents of the Kjeldahl flask (*see* **17.2.1**) and transfer the contents to a 600-ml beaker. Rinse the Kjeldahl flask three times with boiling water, transfer the washings to the beaker to make a total volume of about 400 ml. Cool the contents of the beaker, add ammonium hydroxide solution until the contents are just alkaline, then add 20 ml of either concentrated hydrochloric acid or dilute sulphuric acid. Cover the beaker with a watch glass, heat the solution contained in the beaker to about 95°C and pass slow stream of hydrogen sulphide gas through the solution for one hour. Digest this mixture at about 95°C for one hour and allow it to stand for 30 minutes. Filter the contents of the beaker through a quantitative filter paper and wash the residue of stannous sulphide on the filter alternately with three portions each of wash solution and hot water. Transfer the residue, along with the filter paper, to a 50-ml beaker, add 10 to 20 ml of ammonium polysulphide solution, heat the contents of the beaker to boiling and filter by decantation. Add 10 ml of ammonium polysulphide solution to the contents of the beaker, just bring to boil and filter again in the same manner. Repeat this once more. Finally wash the filter with hot water. Acidify the combined filtrate and washings with dilute acetic acid, gently boil for one hour and allow to stand overnight. Filter resulting mixture through a double 11-cm ashless filter paper. Wash the filter alternately with two portions each of the wash solution and hot water. Transfer the residue along with the filter paper to a tared porcelain crucible and dry it thoroughly in an air oven. Carefully ignite the filter paper, using a Bunsen flame, and incinerate the contents to convert the sulphide to oxide. Then partly cover the crucible and heat strongly over a large Bunsen or Meker burner. (The sulphide shall be gently roasted to the oxide, which may then be heated to a high temperature without loss by volatilization.) Cool the crucible in a desiccator, weigh and find the mass of the residue. Repeat heating strongly, cooling and weighing until the mass of crucible and its contents remain constant.

17.3 Calculation

$$\text{Tin (as Sn), parts per million} = \frac{787\,700\,m}{M}$$

where

m = mass in g of the residue (SnO_2) left in the porcelain crucible (*see* **17.2.2**), and

M = mass in g of the prepared sample taken for the experiment.

18. DETERMINATION OF ZINC

18.0 Principle — This method involves wet oxidation of the material, elimination of lead, copper, cadmium, bismuth, antimony, tin, mercury and silver as sulphides, using copper sulphate solution as scavanger agent, simultaneous elimination of cobalt and nickel by extracting metal complexes of α-nitroso-β-naphthol and dimethyl glyoxime respectively with chloroform, extraction of the zinc dithizonate with carbon tetrachloride, transfer of zinc to dilute hydrochloric acid, and final extraction of zinc dithizonate for colour measurement.

18.1 Apparatus

18.1.1 *Spectrophotometer* — a suitable type.

18.2 Reagents

18.2.1 *Water* — Redistil the water from all resistant glassware, which is thoroughly cleaned with hot concentrated nitric acid.

18.2.2 *Concentrated Nitric Acid* — redistilled, sp gr 1·42.

18.2.3 *Concentrated Sulphuric Acid* — sp gr 1·84.

18.2.4 *Perchloric Acid* ($HClO_4$)

18.2.5 *Methyl Red Indicator Solution* — one percent (*m/v*), aqueous.

18.2.6 *Copper Sulphate Solution* — Dissolve 8 g of copper sulphate ($CuSO_4.5H_2O$) in water and dilute to one litre. One millilitre of this solution contains 2 mg of copper (Cu).

18.2.7 *Ammonium Hydroxide Solution* — redistilled, sp gr 0·90.

18.2.8 *Concentrated Hydrochloric Acid* — sp gr 1·16.

18.2.9 *Hydrogen Sulphide Gas* — from the generator, passed through a wash bottle containing water.

18.2.10 *Dilute Hydrochloric Acid* — (a) 5 percent (*m/v*), (b) 1 : 1 (*v/v*), and (c) 0·04 N approximately.

18.2.11 *Bromine Water* — saturated.

18.2.12 *Phenol Red Indicator Solution* — 0·04 percent (*w/v*). Dissolve 0·1 g of phenol red in 28·2 ml of 0·01 N sodium hydroxide solution and dilute to 250 ml with water.

18.2.13 *Dithizone (Diphenyl Thiocarbazone) Solution* — Dissolve 0·05 g of dithizone in 2 ml of ammonium hydroxide solution and 100 ml of water, and extract repeatedly with carbon tetrachloride until solvent layer is clear and of bright green colour. Discard the solvent layer and filter the aqueous portion through a washed ashless paper. (This solution is best prepared as needed since it is only moderately stable, even when kept in the dark and under refrigeration.)

18.2.14 *Carbon Tetrachloride* — redistilled.

18.2.15 *Chloroform* — redistilled.

18.2.16 *Ammonium Citrate Solution* — Dissolve 225 g of ammonium citrate [$(NH_4)_2HC_6H_5O_7$] in water, make alkaline to phenol red with ammonium hydroxide (*p*H 7·4 first distinct colour change) and add 75 ml in excess. Dilute to 2 litres. Extract this solution immediately before use as given under **18.2.16.1**.

18.2.16.1 Add to the ammonium citrate solution (*see* **18.2.16**) a slight excess of dithizone solution and extract with carbon tetrachloride until the solvent layer is clear bright green. Remove the excess of dithizone by repeated extraction with chloroform, and finally extract once more with carbon tetrachloride. (It is essential that excess dithizone is entirely removed, as otherwise zinc will be lost during elimination of cobalt and nickel.)

18.2.17 *Dimethyl Glyoxime Solution* — Dissolve 2 g of dimethyl glyoxime in 10 ml of ammonium hydroxide solution and 200 to 300 ml of water, filter and dilute to one litre.

18.2.18 *α-Nitroso-β-Naphthol Solution* — Dissolve 0·25 g of α-nitroso-β-naphthol in chloroform to 500 ml.

18.2.19 *Stock Solution of Zinc* — Dissolve exactly 0·500 g of pure granulated zinc in a slight excess of dilute hydrochloric acid and dilute to 1 000 ml.

18.2.20 *Standard Solution of Zinc* — At the time of the experiment, dilute 10 ml of the stock solution of zinc (*see* **18.2.19**) to 1 000 ml with hydrochloric acid (0·04 N). One millilitre of this solution contains 5 µg of zinc.

18.3 Procedure

18.3.1 *Preparation of Material* — Accurately weigh into an Erlenmeyer flask of suitable size, a quantity of well mixed sample not exceeding 25 g, estimated to contain 25 to 100 µg of zinc. Add to the prepared sample concentrated nitric acid and heat cautiously until the first vigorous reaction subsides somewhat, then add 2 to 5 ml of concentrated sulphuric acid. Continue the heating, adding, if necessary, more of concentrated nitric acid in small increments to prevent charring until fumes of sulphur trioxide are evolved and the solution becomes clear and almost colourless. Add 0·5 ml of perchloric acid and continue heating until it has been completely removed. Allow the solution to cool and dilute it to about 40 ml. If necessary equipment is available, wet oxidation and subsequent sulphide separation may be advantageously carried out in a small Kjeldahl flask.

18.3.2 *Separation of Sulphide Group* — To the sulphuric acid solution of the material (**18.3.1**) contained in the Erlenmeyer flask add 2 drops of methyl red indicator solution and 1 ml of copper sulphate solution, and neutralize with ammonium hydroxide solution. Add sufficient quantity of concentrated hydrochloric acid to make the solution about 0·15 N with respect to this acid (about 0·75 ml excess in 50 ml solution is satisfactory). The *p*H of the solution at this point should be 1·9 to 2·1 when measured with glass electrode. Pass a stream of hydrogen sulphide gas into the solution until precipitation is complete. Filter the contents of the flask through a fine textured filter paper (Whatman No. 42 or equivalent) that has been previously fitted into the funnel and washed first with dilute hydrochloric acid (5 percent), and then with water. Collect the filtrate in a beaker, and wash the flask and the filter with 3 or 4 small portions of water. Boil the filtrate gently until the odour of hydrogen sulphide can no longer be detected, then add 5 ml of bromine water and continue boiling until bromine has been expelled. Allow the solution to cool, neutralize with ammonium hydroxide solution using phenol red indicator solution, and then make it slightly acidic with hydrochloric acid (1 : 1) by adding an excess of 0·2 ml. Dilute the resultant solution in a volumetric flask to contain 0·2 to 1·0 g of zinc per ml.

18.3.3 *Elimination of Nickel and Cobalt* — Transfer a 20 ml aliquot of the solution (*see* **18.3.2**) to a separating funnel. Add to it 5 ml of ammonium citrate solution, 2 ml of dimethyl glyoxime solution and 10 ml of α-nitroso-β-naphthol solution, and shake the contents of the separating funnel for two minutes. Discard the layer of chloroform and extract the aqueous layer with 10 ml of chloroform to remove residual α-nitroso-β-naphthol. Discard the chloroform layer.

18.3.4 *Isolation and Estimation of Zinc* — To the aqueous layer obtained after eliminating nickel and cobalt (*see* **18.3.3**), which at this point has a *p*H of 8·0 to 8·2, add 2·0 ml of the dithizone solution and 10 ml of carbon tetrachloride, and shake after two minutes. Allow the layers to separate and remove the aqueous layer as completely as possible, withdrawing it by means of a pipette attached to the vacuum line. Wash down the sides of the separating funnel with about 25 ml of water and without shaking again draw out the aqueous layer. Add 25 ml of hydrochloric acid (0·04 N) to the carbon tetrachloride layer in the separating funnel and shake for one minute to transfer the zinc to the acid-aqueous layer. Drain off and discard the carbon tetrachloride layer; being careful to dislodge and remove the drop that usually floats in the surface. To the acid-aqueous layer add 5·0 ml of ammonium citrate solution and 10·0 ml of carbon tetrachloride (*p*H of the solution at this point is 8·8 to 9·0). Determine the volume of dithizone solution to be added as given in **18.3.4.1**.

18.3.4.1 Pipette 4·0 ml standard solution of zinc into a separating funnel, add to it 21 ml of hydrochloric acid (0·04 N) from a burette, 5 ml of ammonium citrate solution and 10·0 ml of carbon tetrachloride and then add dithizone solution in 0·1 ml increments, shaking briefly after each addition until a faint yellow colour in the aqueous layer indicates a bare excess of the reagent. Note the total volume of dithizone solution added. Multiply this volume by 1·5.

18.3.4.2 Add the volume (*see* **18.3.4.1**) of dithizone solution to the solution contained in the separating funnel (*see* **18.3.4**) and shake it for two minutes. Pipette 5·0 ml of the carbon tetrachloride layer and transfer it to the spectrophotometer cell. Dilute the solution with 10·0 ml of carbon tetrachloride, mix and determine its spectral transmission at 540 nm. Dilution may be made in a clean and dry test-tube, if the design of the cell does not permit mixing directly.

18.3.4.3 Pipette into a series of separating funnels, 0, 1, 2, 3 and 4 ml of standard solution of zinc and add the necessary volume of hydrochloric acid (0·04 N) to make 25 ml. Add into each separator 5·0 ml of ammonium citrate solution to the calculated volume of dithizone solution (*see* **18.3.4.1**). Shake the separating funnels for two minutes. From the first separating funnel pipette out 5·0 ml of the carbon tetrachloride layer and transfer it to the spectrophotometer cell. Dilute the solution with 10·0 ml of carbon tetrachloride, mix and determine its spectral transmission at 540 nm. Proceed in the same manner with the solutions contained in other separating funnels.

18.3.4.4 Plot the transmittance on logarithmic scale obtained for each of the series of separating funnels (*see* **18.3.4.3**) against concentration of zinc micrograms present in 25 ml of the diluted standard zinc solution in the particular separating funnel and draw a smooth curve through the points. (Intercept of this curve may vary from day to day, depending on the actual concentration of dithizone used in the final extraction, but the slope of the curve should remain essentially the same.) From the curve obtain the mass of zinc in micrograms present in 25 ml of the acid-aqueous layer (for final extraction of zinc from the material, *see* **18.3.4**).

18.4 Calculation — Zinc, parts per million $= \frac{m\,V}{20\,M}$

where

m = mass in g of zinc present in 25 ml of the acid-aqueous layer prepared from the material (*see* **18.3.4.3**);

V = volume in ml of the solution made up to contain 0·2 to 1·0 g of zinc per ml, prepared after eliminating the sulphide group from the material (*see* **18.3.2**); and

M = mass in g of the prepared sample taken for the experiment (*see* **18.3.1**).

19. GENERAL GUIDELINES FOR TESTING THE ABILITY TO SUPPORT THE GROWTH OF MICRO-ORGANISMS

19.0 Two methods are described below. Any one of them may be followed and the test should be stated in the report.

19.1 Test for the Ability to Support Growth — The ingredient to be tested is incorporated in a suitable culture medium strictly according to the formula prescribed for the medium. The medium selected should be such as to have only the test ingredient as the sole growth supporting reagent. Special care is to be taken to see that the medium so prepared is of the *p*H as in the prescribed formula and is sterile before inoculating with the test organism. In solid medium several test organisms can be inoculated in a culture plate. If the medium is liquid, each strain will be tested in separate tubes of the medium. The inoculation medium is incubated at the appropriate temperature, aerobically or anaerobically as the case may be and for the required period of time depending upon the test organisms used.

19.1.1 Profuseness of growth and its character are used in satisfying that the ingredient under test is suitable for use.

19.2 Alternative Method of Testing the Ability to Support Growth — Grow the test organism in a suitable liquid medium and prepare several 10-fold serial dilutions in sterile distilled water. Prepare a liquid culture medium with the test ingredient as in **19.1** above and with a calibrated pipette, place one drop of each dilution, beginning with the most dilute suspension, into separate tubes of medium. Also inoculate similarly, tubes of liquid medium in which the organism was grown. After appropriate incubation, compare the ability of the test medium to support growth of the highest dilution of the test organism with that in liquid medium in which the organism was originally grown.

20. CHECKING OF ABSENCE OF FERMENTABLE CARBOHYDRATES

20.1 Reagent

20.1.1 *Phenol Sulfonphthalein* (*Phenol Red*) *Solution* — Dissolve 100 mg of phenol sulfonphthalein in 100 ml of alcohol, and filter, if necessary.

20.2 Procedure — To a 2 percent solution in water add sufficient phenol sulfonphthalein solution to give a readable colour, place a Durham fermentation tubes, and autoclave. Inoculate with a loop of 24-hour culture of *Escherichia coli.* Incubate for 48 hours at 37°C. If no acid, or only a trace of the inner tube, and no gas are produced during the incubation, it indicates freedom from fermentable carbohydrates.

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Telephone : 27 01 31 (20 lines) **Telegrams : Manaksanstha**

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